Effects of Buffered Deglycerolization Solutions on the 21-Day Post-Thaw Preservation of Red Blood Cells.

Running Title: Effects of Deglycerolization Buffering.

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ABSTRACT

Fresh-frozen or rejuvenated-frozen red cells were deglycerolized by centrifugal washing. The effects of phosphate buffering of the wash solutions on the pH of the washed cell suspension was examined. Buffering raised the red cell pH, and the positive effects of this higher pH were examined when the cells were re-stored at 4°C for 21 days in either AS-17 or AS-3 preservation solutions. The results indicate that the buffering will improve the ability of AS-17, and to a lesser extent AS-3, to preserve fresh, or rejuvenated, frozen-thawed red cells.

Key Words: blood storage, frozen blood, 2,3-DPG, ATP, post-thaw storage, deglycerolization.

INTRODUCTION

Red cells may be preserved for up to ten years by freezing them at -80°C in the presence of 6-M glycerol. Before the cells can be used, they must be thawed at 37-40°C and washed with 2 L of saline-glucose solution (pH 5.9) to remove the glycerol. The deglycerolized cells are then placed in a refrigerator and, according to FDA requirements, must be used within one day or be discarded. To overcome the one-day storage limit, we have developed post-thaw preservation solutions which allow the cells to be re-stored as long as 21 days at 4°C.[1-3]

One of the factors affecting frozen-thawed red cells is the decrease in pH which occurs during the freeze-thaw process. Red cells freshly drawn in CPDA-1 have a blood pH of 7.05 at 37°C. After freezing, thawing, and deglycerolizing, the cell suspension pH drops to pH 6.6-6.7. Red cells which were stored at 4°C prior to freezing, with or without intervening chemical rejuvenation, have even lower post-thaw pH values in the range of pH 6.4-6.5. These low pH values limit post-thaw storage and encouraged us to develop preservative solutions of higher intrinsic pH than those used in additive solutions for fresh blood.[1-3]

We recently reported[3] on an experimental preservative solution, denoted AS-17, which offers superior function and slightly superior viability over the AS-3. Miles AS-3 is the currently approved additive solution for 35-day, 4°C storage of

never-frozen red cells, which, in our opinion, performs best when adapted to a post-thaw situation. One clear advantage of AS-17 over AS-3 is its higher intrinsic pH of 8.9. Currently available additive solutions designed for never-frozen red cells are all made acidic to stabilize the glucose component during the sterilization step of manufacture.

In this report we present data which show that by buffering the saline-glucose deglycerolizing solution to either pH 7.0 or 7.4 we raise the washed cell pH. This buffering also helps to maintain the pH and other metabolic parameters during 21-day post-thaw storage when using either of two previously studied additive solutions.[3]

MATERIALS & METHODS

Blood Processing

All units of blood were drawn from normal healthy adult volunteers after obtaining informed consent. AABB standards were followed throughout. Blood was collected into CPDA-1 using oversized (800 ml) triple bag sets. Fresh-frozen red cells (FF-RBC) were prepared by freezing the blood within 2 hours of donation by the standard military protocol of Valeri.[4,5] Outdated, banked, CPDA-1 packed red cells (REJ-RBC) were also frozen by the same protocol after rejuvenation with 50 ml of PIPA solution (PIPA Labs, Boston, MA). PIPA solution contains phosphate, inosine, pyruvate, and adenine which regenerate red

cell ATP and 2,3-DPG to fresh-blood levels after 1 hour of incubation at 37°C.

All frozen units were thawed in a 40°C water bath and deglycerolized with a Haemonetics Model 115 cell washer (Braintree, MA) using the military protocol.[5] The wash solution was 0.2% dextrose and 0.9% sodium chloride in 2 L bags (Baxter Healthcare Corp., Deerfield, IL) and had a pH of 5.9 (at 25°C). As indicated, some units were deglycerolized with wash solution which had been pretreated with one of two 50 ml concentrated solutions of mono- and disodium phosphates which buffered the wash solution pH to either 7.0 or 7.4. The sterile, pyrogen-free phosphate solutions were prepared by PIPA Labs, (Boston, MA) and aseptically transferred with a syringe to the bag of wash solution.

Post-Thaw Preservation

Deglycerolized units were centrifuged (2200 rpm x 10 min) to repack the red cells. Most of the supernatant was removed with a plasma press to produce a hematocrit of ≥ 80%. The additive solution was added to the red cells, mixed, and a time-zero sample taken. The unit was then stored at 4°C for 3 weeks and sampled weekly. Each sample, about 12 ml in volume, was used to measure pH, P50, red cell morphology, lysis, red cell ATP and 2,3-DPG by methods previously described.[6,7] Each study arm or group (i.e., preservative solution and/or wash pH) consisted of 5 red cell units. All bags of red cells were cultured at the end of the study to assure sterility. Statistical analyses (t-tests)

were done using an alpha of 0.05 with the NCSS 5.0 statpack (Kaysville, UT).

Two additive solutions were used in this study. One was the Miles Inc. (Covina, CA) AS-3 Nutricel solution consisting of 1.1 g glucose, 0.42g citric acid, 0.276 g monobasic sodium phosphate, 0.410 g sodium chloride, 0.030 g adenine, and 0.588 g sodium citrate in 100 ml of water at pH 5.8. The second solution, designated as AS-17, consisted of 0.46 g dibasic sodium phosphate, 0.56 g sodium citrate, 0.030 g adenine, 1.20 g dextrose, and 0.90 mg of citric acid in 60 ml of water at pH 8.3.

Two categories of red cells were evaluated: fresh-frozen and stored-rejuvenated-frozen. Within each category the AS-3 solution was tested with cells washed (deglycerolized) without buffer (pH 5.9) and with the wash buffered to pH 7.4. The AS-17 solution was evaluated using each category of cells washed without buffer (pH 5.9) or with buffered washing to either pH 7.0 or pH 7.4.

RESULTS

The effects of buffering the deglycerolizing solution on the subsequent extracellular pH of red cells re-stored in AS-3 or AS-17 are shown in Figure 1. When FF-RBC (top) were washed in pH 7.4 buffered glucose-saline then placed in either additive solution the results were about 0.2 pH units higher than when no buffering was used. In the same storage solution, the superior

pH of the buffered (vs nonbuffered) units was maintained for 7 days, but there was no difference by day 14. Initial pH results were higher in AS-17 than in AS-3 solution because of its elevated intrinsic pH (8.3 vs 5.9). Throughout the 21 days, the units stored in AS-17 retained higher pH than those stored in AS-3.

The pH of post-thawed REJ-RBC is shown in the bottom of Fig. 1. Deglycerolized, REJ-RBC have a pH of 6.25-6.30 at 37°C. Suspension of the cells in AS-17 solution increased the pH to 6.45. If the deglycerolization solution was buffered to pH 7.0 or pH 7.4, the resulting initial pH in AS-17 rose to 6.62 or 6.85 respectively. The pH of these suspensions remained significantly higher than the pH of suspensions prepared using unbuffered (pH 6.6) deglycerolizing solution throughout 21 days of storage. All the groups decreased, however, at similar rates between 7 and 21 days. Storage of red cells in AS-3 after deglycerolization at pH 7.4 resulted in significantly lower pH than cells suspended in AS-17. Cells stored in AS-3 without buffered washing had an initial pH of 6.25, and continued to drop thereafter (data not shown).

Figure 2 shows the post-thaw red cell ATP levels for fresh-frozen (top) and rejuvenated red cells (bottom). The ATP of FF-RBC did not vary significantly between buffered and unbuffered washes when stored in AS-17, however when stored in AS-3, buffering did slightly elevate ATP during the final 2 weeks of storage. This increase was not statistically significant.

Raising the deglycerolizing pH with REJ-RBC, which was then restored in AS-17, was more dramatic, increasing the 21-day ATP level from 40 to 70% of normal when nonbuffered was compared to pH 7.4 buffered cells. Storage of pH 7.4 buffered REJ-RBC in AS-3 was significantly less effective than in AS-17 for ATP maintenance.

Post-thaw 2,3-DPG maintenance is shown in Fig. 3. For FF-RBC the 2,3-DPG was maintained significantly better in AS-17 than in AS-3. Buffering to pH 7.4 showed significant improvement compared with no buffering in AS-17 samples, but not in AS-3 samples. Post-thaw storage of REJ-RBC showed that buffering at either pH 7.0 or pH 7.4 significantly improved 2,3-DPG maintenance over no buffering in AS-17. Buffered REJ-RBC stored in AS-3 behaved similarly to unbuffered cells stored in AS-17.

All red cell units studied remained sterile throughout the post-thaw storage period. Freeze-thaw recovery of red cells averaged 93.5 ±2.2% (X±SD) for FF-RBC and 92.3±3.1% for REJ-RBC.

Assays for lysis, morphology index, and P50 are shown for FF-RBC in Table 1. Buffering the wash solution caused an increase in lysis, but all values were still below the 1.0% limit. Buffering also tended to raise the morphology index of the red cells, although the differences in the AS-3 set were not significant. The P-50 data agreed with the 2,3-DPG data in that higher 2,3-DPG values produced higher P-50s. The high mean for AS-17/pH7.4 at day 14 seemed abnormal and was affected by two high outliers in this data set. The P-50 of AS-17/pH7.4 was

significantly higher than the AS-3/pH7.4 red cells on days 7, 14, and 21.

Table 2 shows the REJ-RBC data for lysis, P-50 and morphology index. As for FF-RBC, buffering caused an increase in red cell lysis, which rose to 0.45% by 21 days in AS-17/pH7.4. Storage in AS-3/pH7.4 caused lysis above 1.0% by day 14. Increasing the pH of the deglycerolization of rejuvenated cells again caused a rise in the morphology index and the P50 of the AS-17 stored cells.

DISCUSSION

The mechanical aspects of freezing and thawing red cells has been well documented for over 20 years. However, the high costs (3-4 times that of nonfrozen red cells) and the limited shelf life (24-hours post-thaw) have negated the utility of freezing. The development of post-thaw preservation systems and the ability to salvage outdated O-negative units via rejuvenation will greatly enhance the utility of freezing red cells. The freezing and thawing of red cells does cause several cellular changes, which do not initially alter viability or function, but which do cause the cellular metabolism to "run down" faster at 4°C than never-frozen red cells. These freeze-thaw changes include subtle membrane modifications, a lowered pH, loss of some diffusible metabolites, and loss of external buffering capacity. Because of these changes, post-thawed red cells preserve poorly in most

additive solutions designed for nonfrozen red cell storage. Thus, our studies have focused on re-optimizing additive solutions for post-thaw storage.with particular emphasis on elevating the pH which was depressed during the freeze-thaw process.

In this report we showed that by buffering the saline-glucose deglycerolization solution to raise its pH from 5.9 to pH 7.0 or 7.4, we could significantly raise the pH of the washed red cells above that otherwise obtained when resuspending them in AS-3 or AS-17. In addition, the elevated initial pH resulted in better preservation of cellular ATP, 2,3-DPG, and morphology during the 21-day post-thaw storage.

This study was started to address the low pH of REJ-RBC which have a pH of 6.30 after washing. Outdated red cells have a pH of 6.4 to 6.5 after 35 days of refrigerator storage. Rejuvenation with PIPA does raise the concentrations of ATP and 2,3-DPG to fresh blood levels but does not raise the pH. There is a further drop in pH due to the freeze-thaw process, which results in the pH of 6.3.

AS-3 did not raise this pH and thus, without buffering, is not acceptable as a preservative solution for rejuvenated red cells. Even after suspension in AS-17, the REJ-RBC had a pH of 6.45 which was too low a starting point for post-thaw preservation. If the deglycerolization solution was buffered to pH 7.4, the pH rose to a level where AS-3 could be considered as a preservative solution, but we found that AS-17 was clearly

superior as a REJ-RBC preservative.

When the concept of buffered deglycerolization was applied to FF-RBC post-thaw storage in either AS-3 or AS-17 solutions, we observed that buffering did produce increases in initial pH, small but significant rises in 2,3-DPG, and improved cell morphology. ATP levels were not appreciably changed by buffering and the effect on viability may be negligible, but should be checked by in vivo tests. Our in vitro studies showed a clear metabolic advantage for buffered washing plus AS-17 compared with using AS-3 with or without buffering.

The systems described in this report should be evaluated in vivo for their effects on red cell viability. We need to determine if the buffering improves the viability beyond the times we have reported for unbuffered AS-17.[3] We also need to define the time limits of storage of REJ-RBC when using buffering and AS-17.

In summary, buffering the deglycerolization saline-glucose solutions raises the pH of the washed red cells. This rise in pH then results in improved in vitro storage parameters, particularly when used with AS-17. These modifications predict improved viability and functional properties during post-thaw preservation for both fresh-frozen and rejuvenated-frozen red cells.

TABLE 1

SUPPORTING DATA FOR POST-THAW STORAGE OF FRESH-FROZEN RBC

P-T SOLUTION & WASH pH	DAY 0	DAY 7	DAY 14	DAY 21
		% RBC LYSIS		
AS-17/pH6.6	0.00	0.07±.03	0.16±.05	0.26±0.08
AS-17/pH7.4	0.00	0.20±0.06	0.35±0.14	0.61±0.31
AS-3/pH6.6	0.00	0.06±0.02	0.14±0.05	0.32±0.16
AS-3/pH7.4	0.00	0.15±0.06	0.40±0.22	0.85±0.58
		P-50 IN mm Hg		
AS-17/pH6.6	25±0.4	19±0.4	18±0.2	18±0.2
AS-17/pH7.4	26±0.2	25±0.8	25±2.1	20±1.2
AS-3/pH6.6	25±0.1	NA	16±0.4	16±0.4
AS-3/pH7.4	27±0.5	21±1.0	19±0.4	16±0.2
		% MORPHOLOGY INDEX		
AS-17/pH6.6	100±0	83±3	73±2	62±3
AS-17/pH7.4	100±0	90±1	81±3	77±3
AS-3/pH6.6	100±0.2	83±4	77±5	77±4
AS-3/pH7.4	100±0.2	87±4	83±5	78±5

ALL VALUES ARE MEAN ±SEM, N=5 FOR EACH GROUP.

TABLE 2

SUPPORTING DATA FOR POST-THAW STORAGE OF REJUVENATED-RBC

P-T SOLUTION & WASH pH	DAY 0	DAY 7	DAY 14	DAY 21
		% RBC LYSIS		
AS-17/pH6.6	0.00	0.09±.03	0.19±.08	0.34±0.15
AS-17/pH7.0	0.00	0.01±0.02	0.20±0.03	0.29±0.05
AS-17/pH7.4	0.00	0.18±0.04	0.33±0.07	0.45±0.08
AS-3/pH7.4	0.00	0.73±0.22	1.06±0.24	1.45±0.27
		P-50 IN mm Hg		
AS-17/pH6.6	25±1	22±2	17±1	15±0.3
AS-17/pH7.0	NA*	NA	NA	NA
AS-17/pH7.4	32±1	29±1	23±1	21±1
AS-3/pH7.4	32±1	29±1	21±1	18±1
		% MORPHOLOGY INDEX		
AS-17/pH6.6	98±0.3	94±2	73±6	59±5
AS-17/pH7.0	98±1	92±8	79±7	73±4
AS-17/pH7.4	99±1	96±1	92±2	88±2
AS-3/pH7.4	99±3	96±1	92±2	78±2

NA* P-50 NOT RUN ON THIS SET DUE TO INSTRUMENT MALFUNCTION.

ALL VALUES ARE MEAN ±SEM, N=5 IN EACH GROUP.

Figure Captions

Figure 1.

The pH of red cell suspensions measured during post-thaw preservation at 4°C. The pH was measured at 37°C in a Corning Blood Gas Analyser. N=5 for each group.

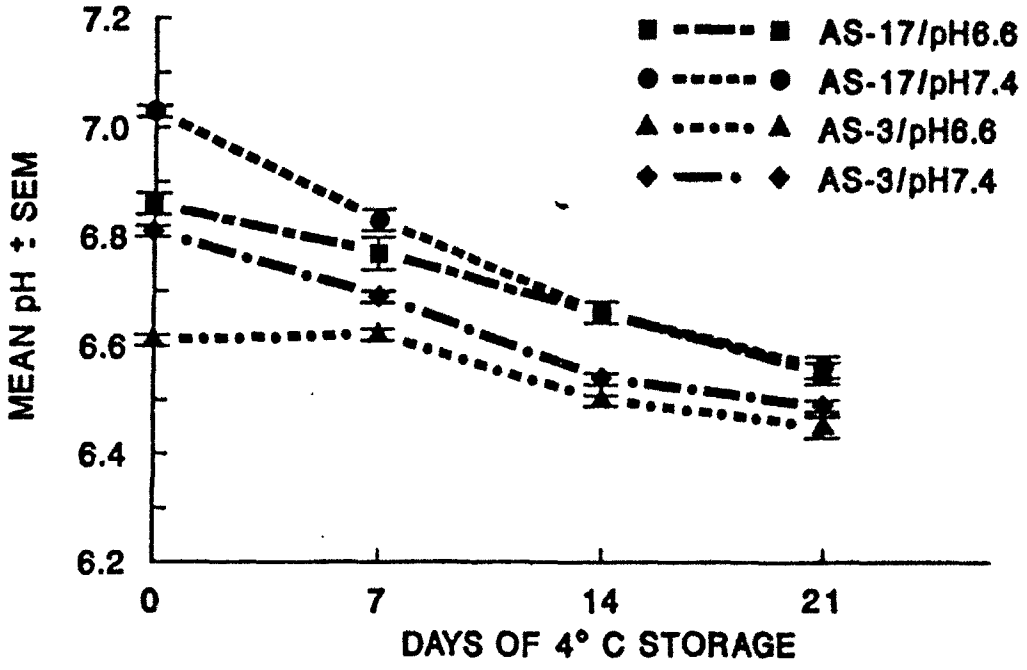
Figure 2.

Red cell ATP levels during post-thaw preservation. Values expressed as a percent of the time-zero value. Time zero values were in the normal range of 4.0±0.5 micromoles/g hemoglobin.

Figure 3.

Red cell 2,3-DPG levels during post-thaw preservation. Values expressed as a percent of the time-zero value. Time zero values were in the normal range of 12.0±1.5 micromoles/g hemoglobin.

FRESH-FROZEN CELLS



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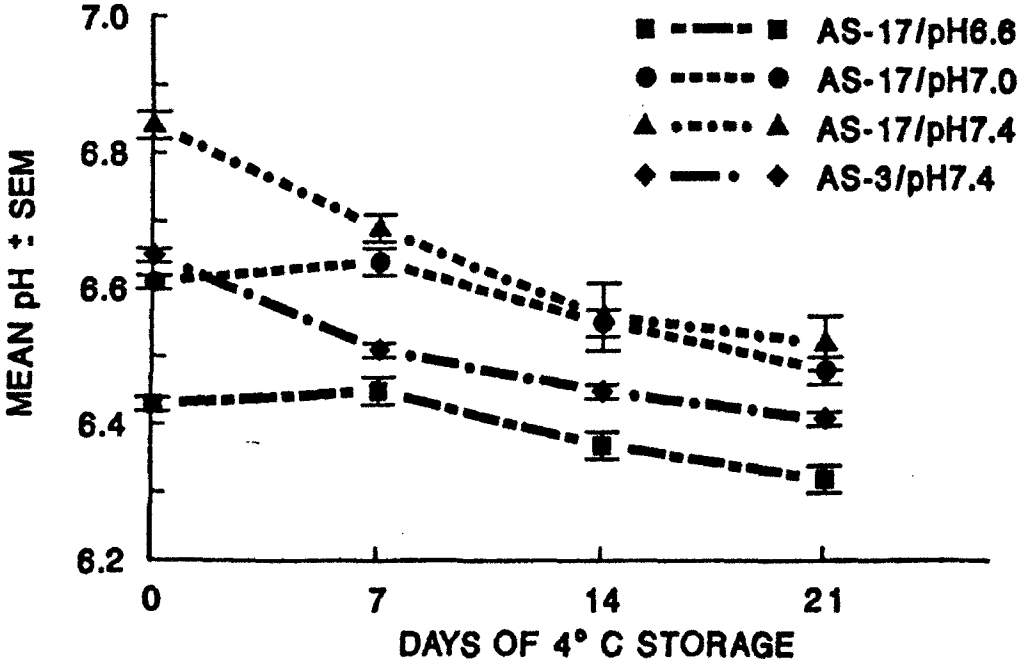

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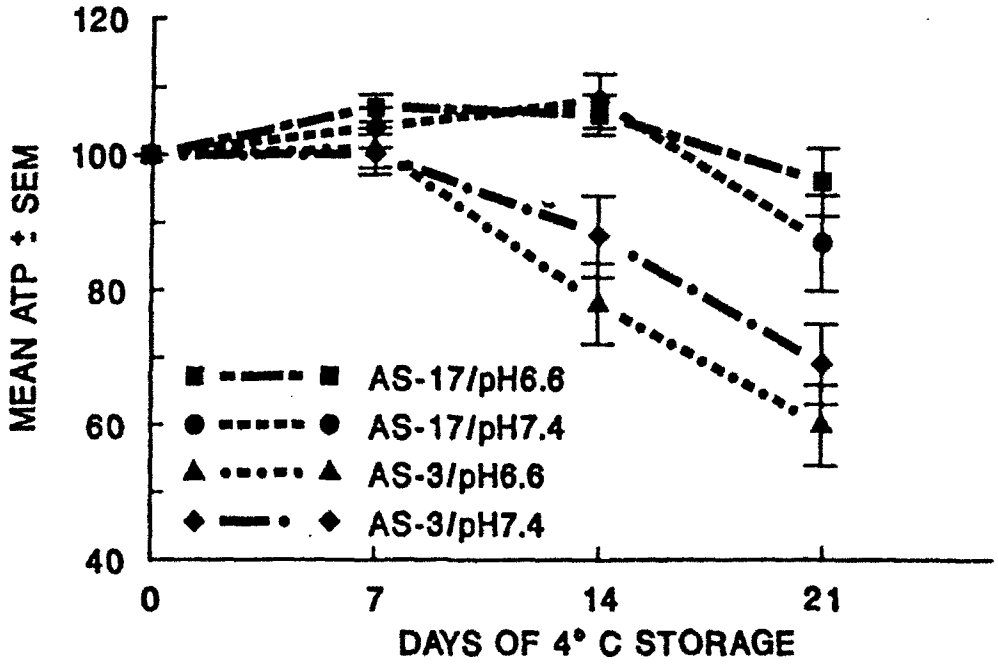

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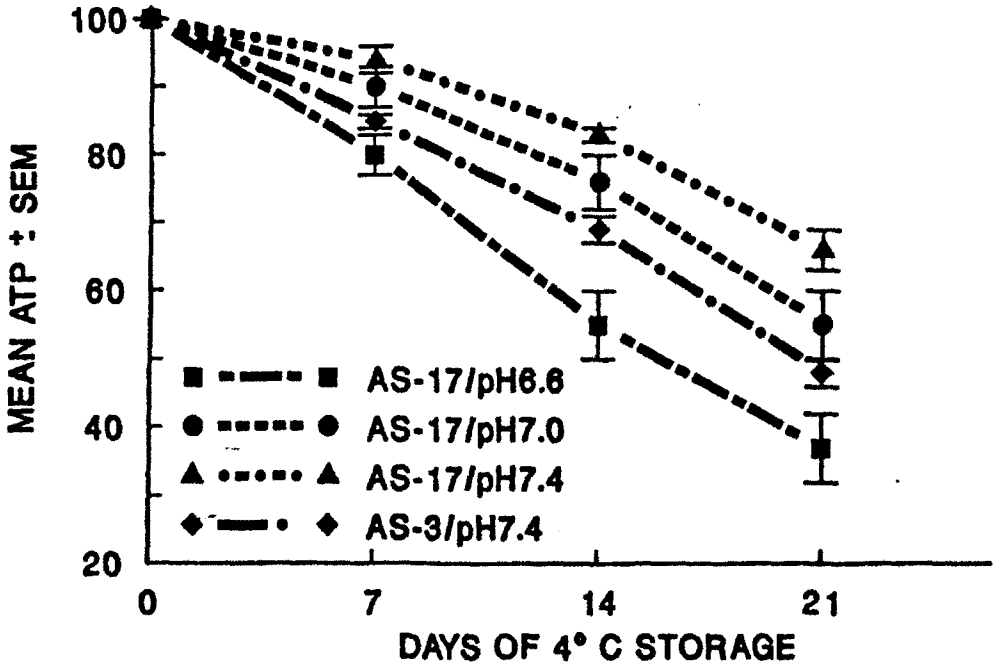

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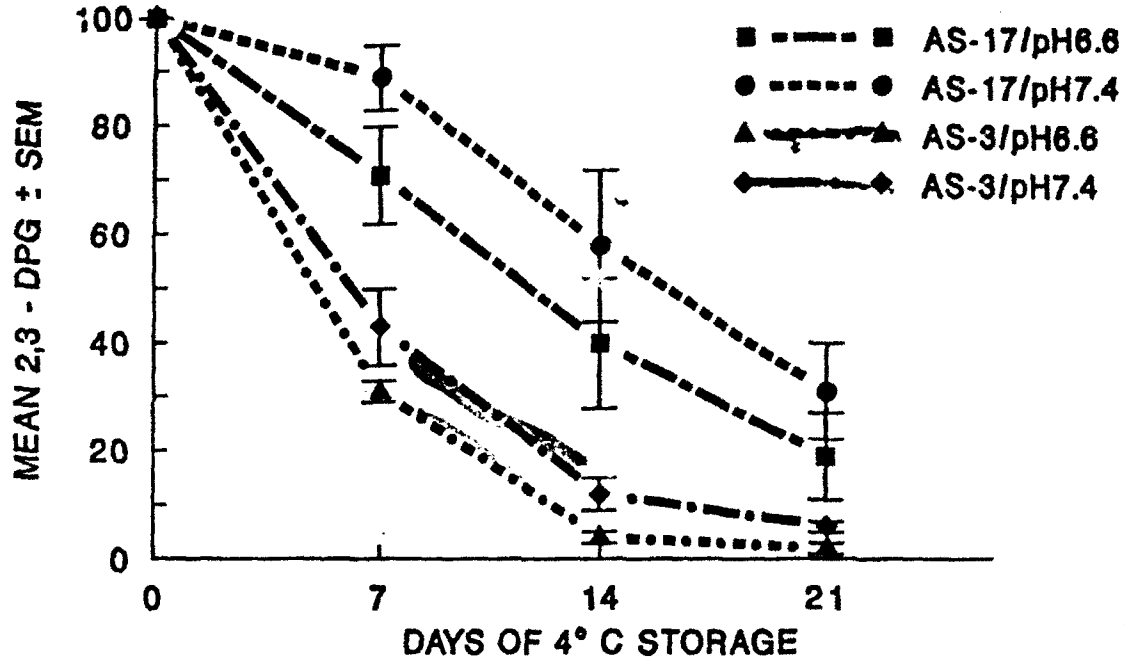

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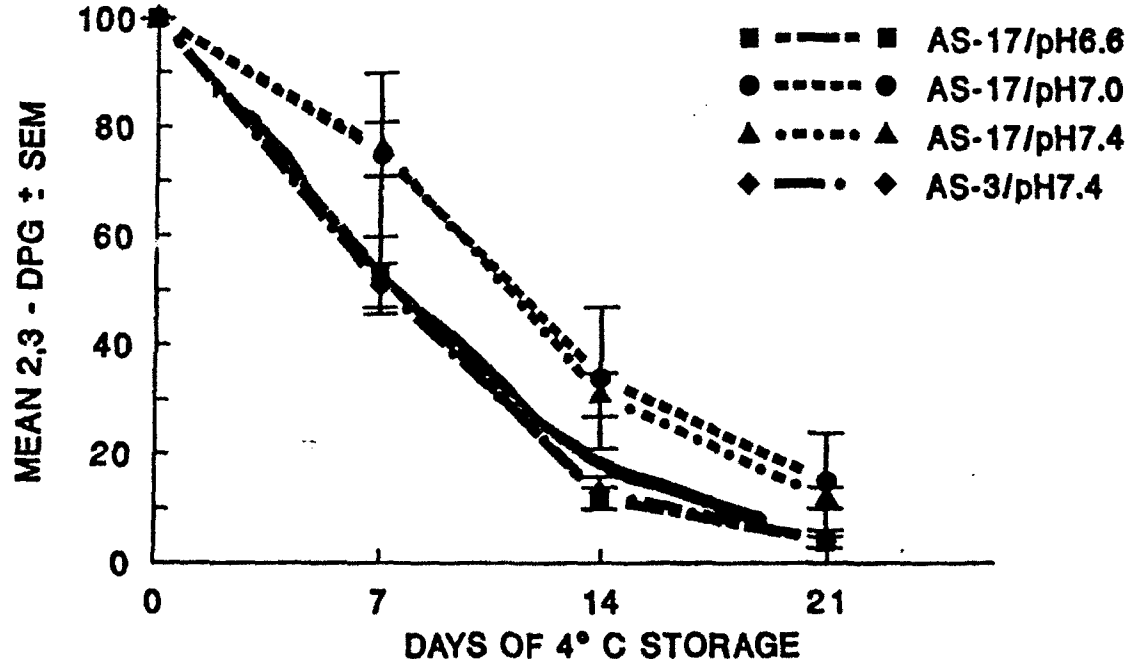

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